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Evaluation of a *Pyrenophora teres* f. *teres* mapping population reveals multiple independent interactions with a region of barley chromosome 6H ☆

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ABSTRACT

The necrotrophic fungal pathogen *Pyrenophora teres* f. *teres* causes the foliar disease net form net blotch (NFNB) on barley. To investigate the genetics of virulence in the barley- *P. teres* f. *teres* pathosystem, we evaluated 118 progeny derived from a cross between the California isolates 15A and 6A on the barley lines Rika and Kombar, chosen based on their differential reactions to isolates 15A and 6A for NFNB disease. Genetic maps generated with SNP, SSR, and AFLP markers were scanned for quantitative trait loci (QTL) associated with virulence in *P. teres* f. *teres*. Loci underlying two major QTL, *VR1* and *VR2*, were associated with virulence on Rika barley, accounting for 35% and 20% of the disease reaction type variation, respectively. Two different loci, *VK1* and *VK2*, were shown to underlie two major QTL associated with virulence on Kombar barley accounting for 26% and 19% of the disease reaction type variation, respectively. Progeny isolates harboring *VK1*, *VK2*, or *VR2* alone were inoculated onto a Rika × Kombar recombinant inbred line mapping population and the susceptibility induced by each pathogen genotype corresponded to the same region on barley chromosome 6H as that identified for the parental isolates 15A and 6A. The data presented here indicate that the *P. teres* f. *teres* – barley interaction can at least partially be explained by pathogen-produced necrotrophic effectors (NEs) that interact with dominant barley susceptibility genes resulting in NE triggered susceptibility (NETS).

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1. Introduction

Pyrenophora teres. f. *teres* is an ascomycete that causes the foliar disease net form net blotch (NFNB) on barley. NFNB received it's name because of the net-like lesions that developed after the pathogen had penetrated and colonized the leaves of susceptible barley lines (Atanasoff and Johnson, 1920; Shipton et al., 1973). Although the disease is most severe in regions of the world with temperate climate paired with high rainfall and high humidity, it can also occur in regions with a variety of temperatures as well as where little rainfall is present (Steffenson and Webster, 1992). Outbreaks of NFNB often produce yield losses of 10–40% by means of reduced kernel size, although the entire crop can be destroyed in extreme situations where the environment is ideal for the pathogen to infect a susceptible host (Mathre, 1997).

P. teres. f. *teres* is closely related to other Dothideomycete genera in the Pleosporales including *Cochliobolus, Parastagonospora,* and *Alternaria*, some of which contain multiple species that produce host selective/specific toxins or necrotrophic effectors (NEs) to induce disease on their specific hosts (Stergiopoulos et al., 2013; Wolpert et al., 2002). Early work on NFNB showed that the *P. teres* f. *teres* – barley system was complex. On the host side, dominant, incompletely dominant, and recessive genes for control of resistance have been reported (Reviewed in Liu et al., 2011). On the pathogen side, pathotypes that were cultivar-specific were identified, indicating strong host genotype specificity (Khan and Boyd, 1969). Subsequently, several studies evaluated virulence diversity in the pathogen by inoculating pathogen populations from specific geographical regions on sets of barley lines that dif-

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fered for resistance/susceptibility (Arabi et al., 2003; Cromey and Parks, 2003; Gupta and Loughman, 2001; Jalli, 2004; Jalli and Robinson, 2000; Jonsson et al., 1997; Khan, 1982; Liu et al., 2012; Sato and Takeda, 1993; Steffenson and Webster, 1992; Tekauz, 1990; Wu et al., 2003). These virulence studies showed high levels of virulence diversity both within a region and worldwide, with some studies indicating strong selection pressure on the pathogen (reviewed in Liu et al., 2011).

Resistance/susceptibility in the NFNB system has been shown to be both qualitative and quantitative and the qualitative relationship between the pathogen and host has been proposed to follow a gene-for-gene model (Afanasenko et al., 2007; Friesen et al., 2006a, 2006b; Lai et al., 2007; Weiland et al., 1999), as proposed by Flor (1956). More recent work on the *P. teres* f. *teres* – barley interaction has indicated the potential for toxins, including host selective toxins (NEs) (Sarpeleh et al., 2007, 2008, 2009), and multiple dominant susceptibility genes (Abu Qamar et al., 2008; Liu et al., 2011) to be involved in disease development. The results from these studies point to the possibility that, in addition to a gene-for-gene type interaction, there is also evidence of an inverse gene-for-gene interaction where NEs are interacting with dominant susceptibility gene products to induce cell death similar to the closely related wheat pathogens *P. tritici-repentis* and *Parastagonospora nodorum* (Ciuffetti et al., 2010; Faris et al., 2010, 2013; Friesen et al., 2008; Oliver et al., 2012). In these necrotrophic specialist systems, programed cell death (PCD) is induced when the pathogen-produced NEs interact directly or indirectly with dominant host susceptibility gene products resulting in disease (Faris et al., 2010), opposite of the dominant resistance that is typically found in a biotrophic interaction.

Our lab previously showed that *P. teres* f. *teres* isolate 6A was virulent on the barley line Rika but avirulent on the barley line Kombar. Conversely, *P. teres* f. *teres* isolate 15A was avirulent on Rika but virulent on Kombar (Abu Qamar et al., 2008; Liu et al., 2011). F_2 individuals of a Rika × Kombar cross were inoculated independently with either 6A or 15A, which resulted in a 1:3 resistant:susceptible ratio for each, indicating that a single dominant gene conferred susceptibility to each isolate. Subsequently, we developed a Rika × Kombar recombinant inbred line (RIL) mapping population to locate the genes responsible for susceptibility to these two *P. teres* f. *teres* isolates. The results showed that genes conferring susceptibility to both 6A and 15A were closely linked in a region of barley chromosome 6H, but in repulsion. The genes were separated by two recombination events, showing that at least two genes were conferring susceptibility; one conferring susceptibility to isolate 15A harbored by Kombar and the other conferring susceptibility to isolate 6A harbored by Rika (Abu Qamar et al., 2008). In the current study, we generated a cross of the *P. teres* f. *teres* isolates 6A and 15A to generate robust *P. teres* f. *teres* genetic linkage maps to investigate this interaction. The progenies from the 15A × 6A population were phenotyped on Rika and Kombar to evaluate the genetics of virulence as it relates to the susceptibility genes identified previously.

2. Materials and methods

2.1. Development of a P. teres. f. teres population

P. teres. f. *teres* isolates 15A (Steffenson and Webster, 1992) and 6A (Wu et al., 2003) were collected from different regions in California and have different virulence patterns on the lines used in this study. A population of 118 progeny from a cross between *P. teres* f. *teres* isolates 15A and 6A was created as described by Lai et al. (2007). Briefly, 100 μL of 15A and 6A inoculum (4000 spores/mL diluted in water) was pipetted onto opposite ends of senesced, sterilized wheat stems. Five wheat stems were placed on one plate of Sach's media (1 g $CaNO_3$, 0.25 g $MgSO_47H_2O$, trace $FeCl_3$, 0.25 g K_2HPO, 4 g $CaCO_3$, 20 g Agar, ddH_2O to 1 L) and stored in the dark at 13 °C until fruiting bodies began to develop on the wheat stems (approximately 3 months). Once mature fruiting bodies had emerged, each wheat stem was transferred to the lid of a water agar plate and placed in the same incubator, with the water agar above the wheat stem. The samples were placed in a 12 h light/dark cycle and left to shoot ascospores onto the water agar plate. The ascospores from the cross were collected from the water agar plate that was placed over the pseudothecia covered wheat stem. A single ascospore was isolated from groups of ascospores to avoid clones that had shot from a single ascus. Individually collected ascospores were then allowed to generate sporulating colonies and a small portion of this fungal sample was then spread across a section of water agar so that single conidia could be isolated. Each progeny was transferred to a V8PDA (150 mL V8 juice, 10 g difco PDA, 3 g CaCO3, 10 g agar, ddH_2O to 1 L) plate and progeny were single-spored an additional time by isolating individual conidia to ensure genetic purity of the samples. Pure isolates were stored at −20 °C as 8 mm diameter dried plugs after being grown and collected.

2.2. Inoculation of barley lines

Conidia from 15A, 6A, and the 118 progeny were collected for inoculum to be used on a collection of barley genotypes consisting of Rika, Kombar, Hector, and NDB112. The genotypes Hector and NDB112 served as susceptible and resistant checks, respectively to ensure even inoculations between progeny isolate applications. The inoculum was generated by placing a lyophilized plug on a V8-PDA plate for 5–7 d at 20 °C in a dark cabinet, 24 h at room temperature in the light, followed by a 24 h dark period at 13 °C. After the isolates had grown out and gone through the above light cycles, the plates were flooded with 100 mL of sterile distilled water and gently but vigorously scraped with an inoculating loop. The collected inoculum was then diluted to a concentration of 2000 conidia per mL and three drops from an eyedropper (approximately 30 μL) of Tween 20 was added to the inoculum per every 50 mL of spore suspension. Plants were grown to the 2 to 3-leaf stage, (approximately 14 d) according to Lai et al. (2007). The plants were grown in racks of 49 cone-tainers with a border of 'Robust' barley surrounding the lines to reduce any edge effect. Each rack of plants was inoculated according to Friesen et al. (2006a, 2006b), by using a paint sprayer (Huskey, model # HDS790) for application until each leaf was almost to the point of inoculum runoff. After inoculation, plants were placed in mist chambers for 24 h at 100% relative humidity, a temperature of 21 °C, and a 24 h light cycle. Plants were then moved to a growth chamber under a 12 h photoperiod at 21 °C until evaluation. Seven days after inoculation, the plants were evaluated for disease symptoms and rated according to the Tekauz (1985) scale. A total of three replicates across the barley line set were completed for each parental and progeny isolate with each replicate consisting of two cones with three barley plants per cone that were scored collectively.

2.3. Fungal DNA extraction

For simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) marker development, fungal DNA was extracted from all collected isolates by placing a dried plug on a V8-PDA plate and allowing it to grow in the dark for 7–10 d. After the fungal isolate had covered the plate, the aerial mycelial tissue was removed and placed in a 2 mL microcentrifuge tube before being placed in a lyophilizer overnight. After all samples had been collected and dried, each was ground using a small pestle and

500 μL of lysing solution (Qiagen BioSprint 15 Plant extraction kit) was added to the ground tissue and the solution was vortexed and centrifuged for 5 min at 6000*g*. DNA was extracted using the Qiagen BioSprint 15 Plant extraction kit (200 μL alcohol + 20 μL MagAttract Suspension + 200 μL DNA supernatant, 500 μL RPW buffer, 1000 μL of 100% ethanol split between two wells, and 200 μL sterile water or TE buffer).

For DNA extractions to be used in genotype by sequencing (GBS) library construction, a dried plug of each isolate was grown on a V8-PDA plate in the dark for 7–10 d and then placed in a 24 h light/24 h dark cycle for the production of spores. After the light/dark cycle was completed, a sterile aluminum foil-covered 250 mL flask containing 60 mL of Fries medium [5 g $(NH_4)_2C_4H_4O_6$, 1 g NH_4NO_3, 0.5 g $MgSO_4 * 7H_2O$, 1.3 g KH_2PO_4, 5.48 g $K_2HPO_4 * 3H_2O$, 30 g Sucrose, 1 g Yeast extract, 2 mL trace element stock solution (167 mg LiCl, 107 mg $CuCl * H_2O$, 34 mg H_2MoO_4, 72 mg $MnCl_2 * 4H_2O$, 80 mg $CoCl_2 * 4H_2O$, ddH_2O to 1 L), ddH_2O to 1 L] was poured into the V8-PDA plate until the liquid was covering the mycelial tissue. Using a sterile loop the plate was vigorously scrapped, releasing the spores into the liquid media. The spore media mixture was then poured back into the remaining medium within the flask and covered again with the original aluminum foil. The flask of spores and media was placed in a 27 °C incubator shaking at 100 rpm for 48 h. After 48 h, the flasks were removed from the incubator and the contents of the flask were blended with a Waring blender and a sterile blending cup on high for 15 s. The blended contents were then poured equally into two new flasks of 60 mL of Fries medium and placed back in the incubator for 24 h. The following day the fresh mycelial growth was cleaned and collected; the contents of both flasks were poured into a large beaker covered with miracloth and rinsed 3 times with sterile distilled water. The harvested tissue was then transferred to a *Büchner funnel* with P5 filter paper (Fisher brand) and the vacuum applied until all excess liquid was removed and only a dried mycelial mat remained. This dried mat was placed in aluminum foil and left in the −20 °C freezer for 24 h before being placed in the freeze dryer until completely dry. Mycelial mats were ground to a fine powder using a mortar and pestle with liquid nitrogen. A 2 mL microcentrifuge tube was filled to the 200 μL mark with ground mycelial tissue and 700 μL of Qiagen RLT buffer was added to the tube and mixed with repeated pipetting until the solution was homogenized, then 2 μL of RNase (20 mg/mL) was added to the solution and vortexed followed by centrifugation at 4000*g* for 8 min. The supernatant from each sample was poured into a new 2 mL tube and placed on ice for 10 min after 700 μL of isopropanol was added and the tubes were slowly inverted 20 times. After 10 min on ice, the tubes were centrifuged for 8 min at 12,000*g*. All supernatant was removed from the tube and the pellet that had formed was washed with 500 μL of 95% ethanol and placed in the laminar flow hood until dry. Once dry, the pellet was re-suspended in 600 μL TE buffer and 5 μL RNase (20 mg/mL) and placed in a 37 °C incubator for 30 min. After the incubation time had completed, 600 μL of a phenol:chloroform:isoamyl alcohol (25:24:1) solution was added to each tube and inverted 50 times before being centrifuged for 8 min at 12,000*g*. The aqueous solution located above the formed mat was then collected and placed in a 1.7 mL tube and mixed with 10% of the collected volume of 3 M NaOAc (pH 5.2) and 700 μL isopropanol, the solution was inverted 20 times and centrifuged for 8 min at 14,000 rpm. The formed pellet was washed with 500 μL 95% ethanol and placed in the laminar flow hood for drying before being re-suspended in 50 μL of TE buffer.

2.4. Marker development and mapping

AFLP analysis was done as described in Vos et al. (1995). Briefly, primer combinations used in PCR were complimentary to the adaptor sequences plus two additional selective bases were added at the 3' ends and primers were named according to standard AFLP nomenclature (Vos et al., 1995). A total of forty-one SSR primer sets were tested on the parental isolates 15A and 6A to identify polymorphism. Of the primer sets tested, six were used on the entire 15A × 6A mapping population (Table S1). To do this, 2 μl of fungal template DNA at 10–20 ng/μL was added to 8 μL SSR master mix (1 μL 10 × Biolase buffer, 2 μL dNTPs 1 mM each, 0.3 μL forward primer 10 pmol/μL, 0.3 μL reverse primer 10 pmol/μL, 0.3 μL M13 1 pmol/μL, 0.3 μL Taq polymerase, 3.8 μL ddH_2O). The samples were amplified according to Röder et al. (1998) and then brought down to 4 °C for storage.

After the AFLP and SSR procedures had been completed, 2 μL of Li-cor loading dye was added to each of the samples and approximately 2 μL of the sample was loaded onto a polyacrylamide gel (20 mL RapidGel XL 6% liquid acrylamide (USB Corp.), 20 μL Temed, 120 μL APS) on a LI-COR IR^2 DNA sequencer model 4200 global edition and run for 2 h or until the polymorphic bands were observed. Fragments that were polymorphic between the two parental isolates and progeny were scored according to parental type for subsequent mapping. For AFLPs, markers were named according to the standard *Eco*RI and *Mse*I primers used and amplicon size estimate. SSRs were named based on the primer combination and base pair size estimate according to the marker standard (Table S1).

A genotype-by-sequencing (GBS) approach that involves sequencing of restriction-site associated DNA (RAD) modified from Baird et al. (2008) was used for single nucleotide polymorphism (SNP) discovery. Briefly, DNA was extracted from the 118 progeny isolates and the two parental isolates followed by a double restriction digestion of ~400 ng of fungal genomic DNA using the restriction enzymes *Ape*KI and *Hha*I (New England Biolabs). Bar coded Ion Torrent sequencing adaptors modified to contain *Ape*KI and *Hha*I compatible ends were ligated to the digested gDNA from each isolate individually. Forty different barcodes were used allowing for the multiplexing and parallel sequencing of forty isolates on a single 318 Ion Torrent microprocessor sequencing chip. Adapted gDNA of forty isolates was pooled for each Ion Torrent sequencing library and column purified to remove un-ligated adaptors. These pooled libraries were loaded onto the Pippin Prep size selection instrument (Life Technologies) using a 2% pre-cast gel cassette set to select for 275 bp fragments. The size selected fractions were amplified using 2 μL of the adaptor ligated gDNA as the template. Amplification was performed using the following parameters; 95 °C for 1 min, 6 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s; followed by 72 °C for 5 min. The amplified library was diluted to ~4 pg/μL and 20 μL was used in an emulsion PCR reaction to produce monoclonal Ion sphere particles (ISPs) using the Ion Torrent One Touch 2 System (Life Technologies). The final sequencing reactions were performed on the Ion Torrent PGM (Life Technologies) using 318 microprocessor chips following the manufacturer's standard procedure. Barcode identification for parsing sequencing files was completed utilizing the Ion Torrent sequencing server. Sequence alignment was performed with the Laser gene 10.1 software (DNA Star) using the standard manufactures parameters optimized for Ion Torrent sequence data. The unique "sequence tags" were identified by aligning the sequences from the parental isolates and utilized for "templated assembly" and SNP analysis with all progeny isolates in SeqMan NGen (DNA Star). Unique SNP calls were made if a given progeny isolate had three or more quality reads at >95% sequence identity, indicating that the tag was from a unique locus within the *P. teres* genome and the called SNP had greater than 75% of the progeny with one or the other parental SNP calls at the given location. Additionally, SNP markers with segregation skewed past a 3:1 ratio in either direction were not used in mapping.

Linkage groups were created using MapDisto 1.7.6.5.2.2 (Lorieux, 2012). Using the AFLP, SSR and SNP markers, groups were

created using the "find groups" with a LOD threshold setting of 7.0 and an r-max threshold setting of 0.3 and using the Kosambi mapping function. The "check inversions" and "ripple order" functions were used to obtain the best order of markers. The "drop locus" command was used to evaluate potentially problematic markers and markers expanding an interval by more than 3 cM were dropped one at a time to generate high quality linkage groups. Once linkage groups were assembled, maps were generated.

2.5. QTL analysis

The average values of the three replicates of phenotypic data were surveyed for significant associations with marker loci using the QTL analysis software program QGene v4.0 (Joehanes and Nelson, 2008) as described in Liu et al. (2008). The critical LOD threshold for the population was calculated by conducting a test of 1,000 permutations, and resulted in a critical value of 3.9 representing an experiment-wise threshold at the 0.05 level of probability. Significant QTL were identified using composite interval mapping.

2.6. Selection of progenies containing single QTL for evaluation on the RK barley population

Genotypes for the markers associated with the entire peak of each QTL were identified. Each virulence QTL was isolated in a single progeny based on the marker types spanning the QTL of interest. Unfortunately, no single progeny isolate harbored only the VR1 QTL region. Therefore, the three progeny isolates that isolated *VK1* (progeny isolate 20), *VK2* (progeny isolate 63), and *VR2* (progeny isolate 72) alone were identified and used for inoculations. The progeny isolates were inoculated onto the entire Rika × Kombar barley recombinant inbred population (Abu Qamar et al., 2008).

2.7. Statistical analysis

To test for homogeneity between the three replicates for each barley genotype individually, a Barlett's χ^2 test was used. In this analysis, the value obtained was compared to the χ^2 value at $P = 0.05$ with 2 degrees of freedom. Replicates that were not significantly different at the $P = 0.05$ level were combined for QTL analysis.

A least significant difference (LSD) test was used to look for significant differences between average disease scores when the 15A × 6A *P. teres* f. *teres* population was separated into groups that contained different genotypic pairings. SAS 9.3 (SAS Institute Inc., 2011) was used to perform this test at $\alpha = 0.05$. If the difference between two groups was greater than the LSD value calculated, then there was a significant difference between the two groups.

3. Results

3.1. Genome mapping and QTL analysis

A total of 18 linkage groups (LGs) were identified and ordered from largest to smallest according to map distance (Fig. S1). The 18 LGs were comprised of 468 markers spanning 1,799.77 cM. LGs contained from seven markers (LG17 and LG18) to 71 markers (LG2) (Fig. S1). Given that the size of the *P. teres* f. *teres* genome is approximately 42 Mb (Ellwood et al., 2010), the physical to genetic distance ratio in this population is approximately 23 kb/cM, which should allow for efficient map-based cloning of most genes.

Virulence loci were identified using the average of the three replicates performed as inoculations on the barley genotypes Rika and Kombar. Using Barlett's χ^2 test, all three replicates were shown to be homogeneous ($P = 0.05$) on barley lines Rika ($\chi^{2(2df)} = 1.6$) and Kombar ($\chi^{2(2df)} = 3.41$) and were therefore pooled for analysis.

3.2. Genetic analysis of virulence on barley line Kombar

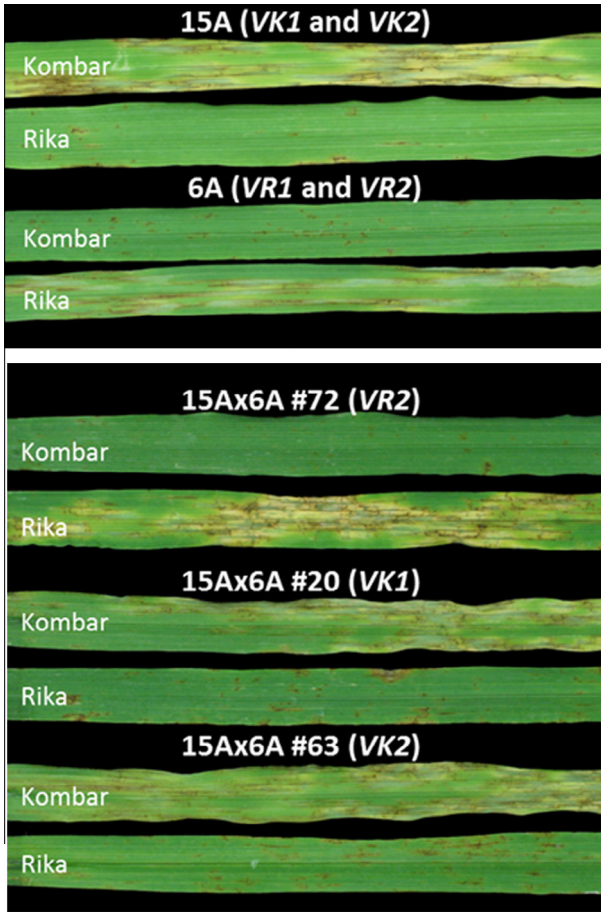
Kombar, on which 15A was virulent and 6A was avirulent (Fig. 1), was used to identify two major virulence loci. The first virulence locus, *VK1* (Fig. 2), was found on LG1 (Fig. 2) and had an R^2 value of 0.26 (Table 1). The second virulence locus, *VK2* (Fig. 2), was found on LG2 and had an R^2 value of 0.19 (Table 1). A least significant difference (LSD) test was performed on the phenotypic data from the four genotypic classes of the 15A × 6A progeny including *VK1*/*VK2*, *VK1*/*vk2*, *vk1*/*VK2*, and *vk1*/*vk2* (Table 2). The LSD analysis showed that there was a significant difference between the genotypic classes that had both virulence loci (i.e. *VK1*/*VK2*) and genotypes that had either *VK1* or *VK2* (i.e. *VK1*/*vk2* or *vk1*/*VK2*). There was also a significant difference between genotypes harboring neither virulence locus (*vk1*/*vk2*) and those genotypic classes harboring one or the other (i.e. *VK1*/*vk2* or *vk1*/*VK2*), however there was no significant difference between genotypic classes harboring only one of the virulence loci (i.e. *VK1*/*vk2* or *vk1*/*VK2*) indicating that the contribution of the two loci was not significantly different (Table 2).

3.3. Genetic analysis of virulence on barley line Rika

Rika, on which 6A was virulent and 15A was avirulent (Fig. 1), was used to identify two virulence loci, both of which were different from those identified on Kombar. *VR1* (Fig. 2) was located on LG2 (Figs. 2 and S1) and had an R^2 value of 0.35 (Table 1). *VR2* (Fig. 2) was located on LG10 (Fig. 2) and had an R^2 value of 0.20 (Table 1). An LSD test was performed on the phenotypic data from the four genotypic classes of the 15A × 6A progeny including *VR1*/*VR2*, *VR1*/*vr2*, *vr1*/*VR2*, and *vr1*/*vr2* (Table 2). The LSD analysis showed that there was no significant difference between the genotypic classes that had both virulence loci (i.e. *VR1* and *VR2*) and the genotypic class that had only *VR1*, the more significant of the two virulence loci identified in inoculations on Rika. There was, however, a significant difference between the genotypic classes harboring only one of the virulence loci (i.e. *VR1*/*vr2* vs *vr1*/*VR2*) (Table 2). There was also a significant difference between the genotypic class harboring neither QTL (*vr1*/*vr2*) and those genotypic classes harboring only one of the virulence loci (i.e. *VR1*/*vr2*, or *vr1*/*VR2*) indicating that the virulence gene underlying each QTL is independently contributing a significant amount to disease (Table 2).

3.4. Isolation of single QTL progeny isolates for evaluation on the RK barley population

Marker data were analyzed to identify 15A × 6A progeny isolates that harbored only one of the four identified virulence loci. Progeny isolate 20 harbored *VK1* alone, progeny isolate 63 harbored *VK2* alone, and progeny isolate 72 harbored *VR2* alone (Table 3). No progeny isolate that harbored only *VR1* was identified. When inoculating the RK RIL population, susceptibility to each of these progeny isolates mapped to the same 6H region that was previously shown to harbor the recessive resistance (dominant susceptibility) genes *rpt.r* and *rpt.k* (Abu Qamar et al., 2008) (Fig. 1). The same Kombar 6H locus shown previously to confer susceptibility to parental isolate 15A (Abu Qamar et al., 2008) accounted for 65% and 74% of the disease variation when the RK population was inoculated with progeny isolates 20 (*VK1*) and 63 (*VK2*), respectively (Fig. 1; Table 3). The same Rika 6H locus shown previously to confer susceptibility to parental isolate 6A (Abu



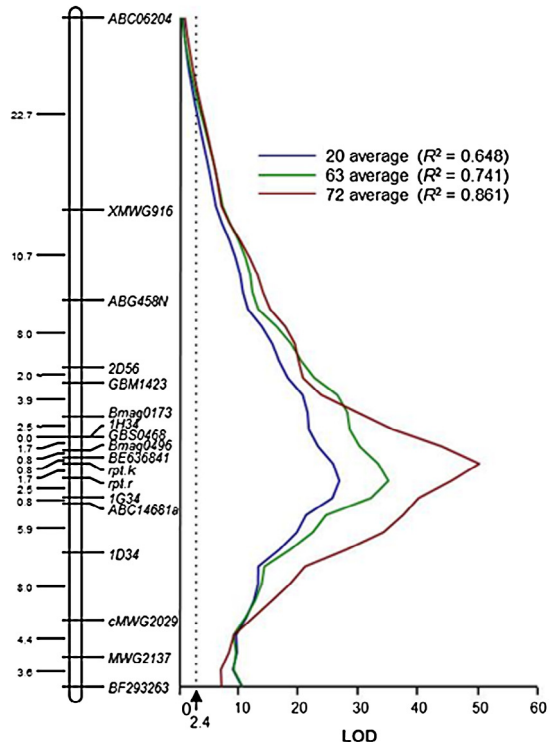


Fig. 1. Top panel – Disease reactions of parental isolates 15A and 6A on barley lines Rika and Kombar. 15A harbors virulence genes *VK1* and *VK2*, both of which confer virulence on Kombar. 6A harbors virulence genes *VR1* and *VR2* both of which confer virulence on Rika. Middle panel – disease reactions of the three single gene progeny isolates on Rika and Kombar. Bottom panel – QTL analysis using the three single progeny isolates showing that the three QTL peak over the susceptibility genes *rpt.k* and *rpt.r* reported in Abu Qamar et al. (2008).

Qamar et al., 2008) accounted for 87% of the disease variation when the RK population was inoculated with progeny isolate 72 (*VR2*) (Fig. 1; Table 3).

This result indicates that the barley 6H region of Kombar likely harbors at least two genes conferring susceptibility to 15A corresponding to *VK1* and *VK2* and Rika harbors at least one gene at the same 6H region conferring susceptibility to 6A corresponding to *VR2* (Fig. 1). Unfortunately, a progeny genotype that could confidently be said to harbor *VR1* alone was not identified in the 15A × 6A population of 118 isolates and therefore the *VR1* interaction with the 6H locus could not be validated. However, given the high significance of *VR1* in virulence we would postulate that the *VR1* gene also interacts with the same 6H region, given the inoculation results of the parental isolate 6A (Abu Qamar et al., 2008).

4. Discussion

Although several NFNB papers have been published describing the genetics of both pathogen virulence/avirulence and host resistance/susceptibility, relative to model interactions, very little is known about how *P. teres* f. *teres* and barley interact, how disease is induced, and how the host effectively resists the pathogen. Both gene-for-gene (effector triggered immunity) and inverse gene-for-gene (necrotrophic effector triggered susceptibility) models have been proposed for the NFNB interaction and it is likely that a complexity of interactions is present. Recent literature describing the host-pathogen interactions involving necrotrophic specialist pathogens such as *P. nodorum*, *Cochliobolus* spp. and the closely related *Pyrenophora tritici-repentis*, have implicated NEs in disease induction. This disease induction has most often involved either proteinaceous or non-proteinaceous effectors being recognized by dominant host genes (Stergiopoulos et al., 2013; Wolpert et al., 2002) leading to signal transduction that results in programmed cell death (PCD). This PCD, although host controlled, does not result in resistance, but conversely, results in disease lesions from which necrotrophic pathogens can gain nutrient. In three cases (Faris et al., 2010; Lorang et al., 2007, 2012; Nagy and Bennetzen, 2008), a dominant host susceptibility gene corresponding to a pathogen produced NE was shown to contain features commonly found in classical resistance genes including nucleotide binding (NB) and leucine rich repeat (LRR) domains. Therefore, there is mounting evidence that necrotrophic specialist fungi often produce NEs that are recognized by the host, leading to a compatible interaction as a result of host-induced PCD.

The global population of *P. teres* f. *teres*, a necrotrophic specialist pathogen, harbors several virulence/avirulence factors, some of which have been mapped and genetically characterized (Reviewed in Liu et al., 2011). In some cases, these factors have been postulated to be avirulence effectors (Affanasenko et al., 2007; Beattie et al., 2007; Lai et al., 2007; Weiland et al., 1999). However, since *P. teres* f. *teres* is a haploid fungus, and none of these genes have been cloned, understanding the nature of virulence/avirulence is as of yet, unclear.

The research findings reported here also cannot determine which pathogen parent is producing the functional gene product that underlies each virulent/avirulent QTL. However, we do know that since host genes conferring susceptibility in both Rika and Kombar are dominant, it is highly likely that this pathogen, at least partially, follows an inverse gene-for-gene or NE triggered susceptibility (NETS) model (Friesen et al., 2007; Liu et al., 2014). In this model, NEs produced by the pathogen elicit host recognition that is followed by defense response signaling that results in hallmarks of a resistance response including increase in active oxygen (Able, 2003; Liu et al., 2014), electrolyte leakage (Liu et al., 2014) and

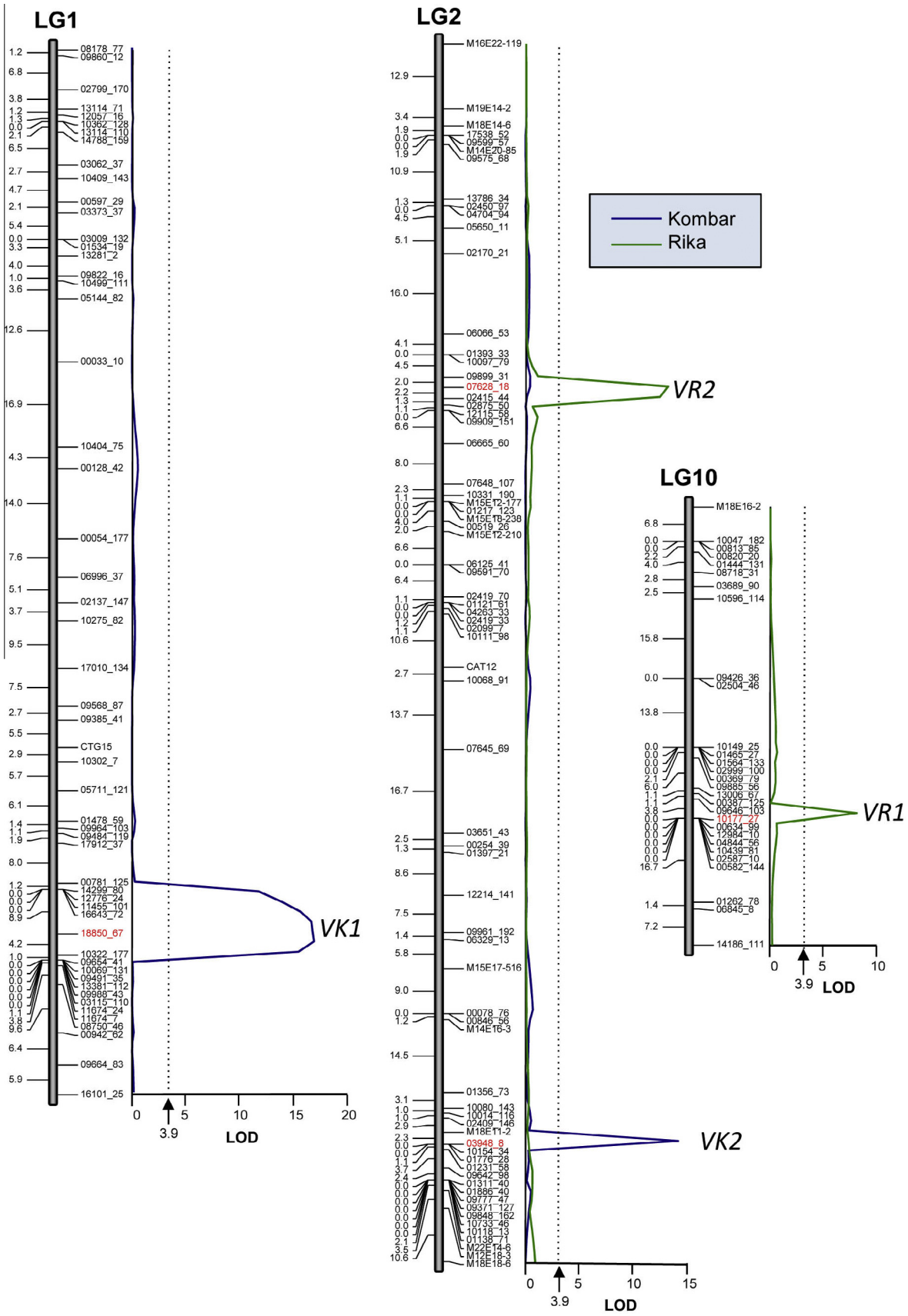


Fig. 2. QTL analysis of virulence to Kombar barley (blue line) and Rika barley (green line) in the *P. teres* f. *teres* population derived from 15A × 6A. The 15A × 6A linkage groups LG1, LG2, and LG10 are shown with the molecular maps to the left and the corresponding QTL composite interval mapping regression curves plotted to the right. On each linkage map, genetic distances in centiMorgans are shown along the left of the maps and markers are shown along the right. The LOD scale is shown at the bottom of the regression curves on the x axis, and the LOD threshold of 3.9 is indicated by the vertical dotted line. The most significant markers for each QTL are also shown in red in Fig. S1.

Table 1
Single marker analysis of virulence QTL identified in the 15A × 6A population. The markers most closely related to *VK1* and *VK2* were compared with phenotypic data from Kombar, while the markers most closely related to *VR1* and *VR2* were compared with phenotypic data from Rika.

Marker	Linkage group	QTL	Rika R^2	Kombar R^2
18850_67	LG1	*VK1*	NS	0.26
03948_8	LG2	*VK2*	NS	0.19
07628_18	LG2	*VR1*	0.35	NS
10177_27	LG10	*VR2*	0.20	NS

NS = non-significant.

Table 2
Average disease reaction types for all possible genotypic classes evaluated for significant differences using a least significant difference (LSD) test. The genotypic class is shown in the first column of each table followed by the number of progeny identified in each genotypic class and average disease reaction type score for progeny included in that genotypic class. The presence of the virulence locus underlying each QTL is shown by capital letters (e.g. *VR1*) and those lacking the virulence locus underlying the virulence QTL are designated by lowercase letters (e.g. *vr1*). Average disease reaction scores that are followed by different letters are significantly different at the $P = 0.05$ level.

Genotypic class	# Of isolates	Average score
A. Rika inoculations		
15A (*vr1*/*vr2*)	–	2
6A (*VR1*/*VR2*)	–	7.2
VR1/*VR2*	34	7.76 **A**
VR1/*vr2*	14	7.43 **A**
vr1/*VR2*	45	6.50 **B**
vr1/*vr2*	20	3.73 **C**
		LSD = 0.6598 ($P = 0.05$)
B. Kombar inoculations		
15A (*VK1*/*VK2*)	–	7.26
6A (*vk1*/*vk2*)	–	1.7
VK1/*VK2*	30	7.38 **A**
VK1/*vk2*	30	6.16 **B**
vk1/*VK2*	25	6.15 **B**
vk1/*vk2*	26	3.35 **C**
		LSD = 0.8189 ($P = 0.05$)

ultimately cell death, all resulting in nutrient for the necrotrophic pathogen.

In the current work, we isolated progeny isolates carrying a single virulence QTL for three of the four virulence QTL identified and showed that susceptibility in the RK population maps to the 6H region as would be expected if NEs were being produced at each of these loci. According to both the ETI and NETS models, if each of the virulence QTL identified in this *P. teres* f. *teres* population represent unique effectors that interact with a unique barley gene product, there should be an equal number of susceptibility/resistance genes in the host as there are virulence/avirulence loci in the pathogen. The virulence factors underlying each of the four QTL were associated with barley lines Rika and Kombar, with *VR1* and *VR2* being associated with virulence on Rika and *VK1* and *VK2* being associated with virulence on Kombar, indicating a total of four different NE genes within the pathogen. From previous research, we know that barley chromosome 6H harbors a region with multiple NFNB resistance/susceptibility genes. Both Rika and Kombar are among the lines to harbor dominant susceptibility genes within this region and more specifically, susceptibility associated with both 15A and 6A map to the 6H region, but in repulsion (Abu Qamar et al., 2008; Liu et al., 2011). These recessive resistance loci (e.g. dominant susceptibility loci) named *rpt.r* and *rpt.k* were separated by two recombinants in a Rika × Kombar RIL population (Abu Qamar et al., 2008). Originally, based on segregation ratios in the host, it was hypothesized that there were only two dominant susceptibility genes on the 6H chromosome region containing *rpt.r* and *rpt.k*, one in Rika conferring susceptibility to 6A and one in Kombar conferring susceptibility to 15A (Abu Qamar et al., 2008; Liu et al., 2011). However, based on the results presented in this study, it is possible that there are four closely linked genes found in the *rpt.r*/*rpt.k* region on barley chromosome 6H that interact with the four *P. teres* f. *teres* NE gene products of *VR1*, *VR2*, *VK1*, and *VK2*.

To characterize the impact on virulence of isolates harboring multiple virulence QTL associated with Rika and Kombar, different progeny genotypes were compared. The genotypes were derived based on the closest markers to the different QTL. For virulence on Kombar, the four genotypic classes included *VK1*/*VK2*, *VK1*/*vk2*, *vk1*/*VK2*, and *vk1*/*vk2*. Genotypes harboring markers coming from the virulent parent 15A for both QTL were significantly different than the other three genotypic classes. Genotypic classes harboring 15A markers for only *VK1* or only *VK2* were not significantly different from one another; however, each of these two genotypic classes was significantly greater than the genotypic class that harbored neither 15A (virulence) QTL. This is yet another strong indication that isolates harboring multiple virulence genes have the ability to cause more severe disease in the presence of the corresponding susceptibility genes in the host.

There is the possibility that a single host susceptibility gene at the 6H locus interacts with both NE products from *VK1* and *VK2* produced by 15A, however, it is equally possible that two distinct 6H host susceptibility targets in Kombar exist but this can only be validated by cloning and characterizing *VK1* and *VK2* and their corresponding host susceptibility target(s). Although these are the simplest explanations for this particular pathogen- host genotype combination, other complex scenarios cannot be ruled out because the literature is filled with examples of both major and minor resistance and susceptibility (Reviewed in Liu et al., 2011).

Somewhat different results were found for the inoculation data collected on the barley line Rika. A significant increase in virulence of progeny harboring both *VR1* and *VR2* was seen when compared to progeny harboring only *VR2*. But, although the disease trend was higher for progeny carrying both *VR1* and *VR2* (7.76) versus that of the progeny carrying *VR1* alone (7.43), there was not a significant difference between *VR1*/*VR2* and *VR1*/*vr2* progeny at the 0.05 level of probability. It is likely that population size and the segregation distortion (Fig. S1 LG 10 and Table 2) involving the *VR2* locus region is at least partially responsible for not identifying a significant difference between these two groups. Additionally, when using a virulence cutoff of 5.0, the virulent:avirulent ratio for progeny isolates is not significantly different than a 3:1 on either Rika or Kombar (data not shown), the opposite of what would be expected in a classical R-gene – Avr-effector interaction. More characterization of this interaction is necessary but it does appear that the NFNB interaction has similarities to the closely related *P. tritici-repentis* and other well characterized necrotrophic fungal interactions such as *P. nodorum* (Oliver et al., 2012) where a NETS interaction has been shown.

In this study, we account for roughly half of the virulence variation observed on these two lines. It is likely that, due to the small size of this population and/or the level of marker saturation, there are other QTL with smaller effects associated with these lines that were not detected and account for the missing heritability. This is evidenced by the fact that when looking at the different genotypic classes of the 15A × 6A progeny, some progeny that contained neither of the markers for virulence on their corresponding host line (seven having *vk1*/*vk2* and four having *vr1*/*vr2* genotypes), still conferred virulent (>5.0) reactions ranging from 5.17 to 8.0. It is also possible that due to recombination between the markers and the actual genes conferring virulence, the genotypic classes do not reflect the actual virulence loci harbored by each progeny isolate. Additionally, even though the phenotyping was not signif-

Table 3
Virulence *VR1* and *VR2* were significant when 15A × 6A progeny are inoculated on Rika, virulence *VK1* and *VK2* were significant when 15A × 6A progeny are inoculated on Kombar, In total, four different virulence QTL where identified, two coming from parental isolate 15A and two coming from parental isolate 6A. Progeny isolates harboring single virulence loci were identified for *VK1*, *VK2*, and *VR2*, however, no progeny isolates were identified that we could confidently say harbored only *VR1*. Bold text represents virulent alleles.

Parents and progeny of the 15A × 6A population	Average disease reactions on Rika	Average disease reactions on Kombar	*VK1* (LG1) genotype	*VK2* (LG2) genotype	*VR2* (LG10) genotype	*VR1* (LG2) genotype
Parental isolate 15A	2.0 (Avirulent)	7.2 (Virulent)	***VK1***	***VK2***	*vr2*	*vr1*
Parental isolate 6A	6.3 (Virulent)	1.6 (Avirulent)	*vk1*	*vk2*	***VR2***	***VR1***
Progeny isolate #20	2.67 (Avirulent)	6.0 (Virulent)	***VK1***	*vk2*	*vr2*	*vr1*
Progeny isolate #63	1.5 (Avirulent)	7.17 (Virulent)	*vk1*	***VK2***	*vr2*	*vr1*
Progeny isolate #72	6.17 (Virulent)	1.0 (Avirulent)	*vk1*	*vk2*	***VR2***	*vr1*

icantly different between replicates, it is possible that some of the remaining variability that was not accounted for by the QTL analysis was due to phenotyping inconsistency between inoculations of individual progeny isolates.

According to the models presented by Jones and Dangl (2006) and Chisholm et al. (2006), pathogens and their hosts are in a constant battle for survival. The host has a network of resistance genes that are effective at recognizing pathogen effectors that are useful in pathogen colonization. Host recognition results in a resistance response involving defense response pathways. The pathogen eludes this defense mechanism through elimination or mutation of these recognized effectors. This model was developed based on research mostly from biotrophic and bacterial systems and does not necessarily apply to pathogens with a necrotrophic lifestyle. In the case of *P. teres* f. *teres*, the pathogen may be using this host gene/effector recognition to its advantage to induce NETS (Liu et al., 2012) rather than ETI. In the necrotrophic pathogen-host plant co-evolutionary model, rather than the host adapting to the presence of the pathogen by evolving resistance genes involved in recognition of pathogen-produced effectors, the pathogen secretes effectors that are "recognized" by the host. Instead of providing a defense for the host, this recognition adds to the offense of the pathogen, allowing the necrotrophic pathogen to take advantage of the released nutrients from the dying tissue (Liu et al., 2012), resulting in proliferation and ultimately sporulation.

The research presented here lays a strong foundation for showing that the *P. teres* f. *teres*-barley interaction is at least partially explained by a NETS model involving dominant host susceptibility genes present at a barley 6H region frequently reported to be involved in NFNB resistance/susceptibility. Previous reports from our group have shown that dominant susceptibility is present at this same 6H region (Abu Qamar et al., 2008; Liu et al., 2011). Additionally, Liu et al. (2014) showed that this 6H region harbors susceptibility to several isolates collected on different continents, as well as sensitivity to NEs identified in intercellular wash fluids of diseased plants. Based on this foundational work, it is now critical that we characterize the genes contributing virulence in the pathogen as well as the corresponding susceptibility genes at the 6H region in barley to get a complete understanding of this host pathogen interaction. This understanding of how *P. teres* f. *teres* is inducing disease is critical to germplasm enhancement aimed at developing NFNB resistant cultivars.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2014.07.012.

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